

# Thermophilic Bacteria Colony Growth and its Consequences in the Food Industry

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## Abstract

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The growth kinetics of thermophilic bacteria *Bacillus acidocaldarius* (CCM 3497), *Bacillus stearothermophilus* (CCMI 237), *Thermus aquaticus* (CCM 3488), *Thermus ruber* (CCM 4212), and *Thermus species* (CCM 4199) on solid media were studied in the temperature range of 40–60°C. The behaviour of bacterial colonies on agar plates was recorded with a digital camera during 160 hours of experiment. The growth of colony diameter was found to be linear with time at lower temperatures and an acceleration of the growth with time at suboptimal growth temperature 60°C was observed. A simple mathematical model describing the effect of irregular colony growth pattern on its rim can elucidate this unusual phenomenon.

**Keywords:** thermophilic bacteria; colony growth; predictive modelling; microbial food spoilage

The growth of bacteria causing spoilage or poisoning of food is generally confined to the surface of foods with solid substrates such as meat, or to the whole volume in liquid substrates such as beverages. Foods are generally able to support the development of diversified microbial consortia, being composed of nutrient-rich materials. The behaviour of microbial populations in foods (growth, survival, or death) can be controlled by the properties of the respective food (e.g. water activity and pH) as well as the storage or technological conditions (e.g. temperature, relative humidity, and atmosphere).

Most studies in food microbiology are concerned with the rapid growth of populations, but in many ecosystems the survival characteristics of the population also need to be considered because the ability of vegetative cells to resist stressful conditions is recognised as an important ecologic trait (RAY 1996). It is well known that the development of microbial contamination is a function of intrinsic food properties (e.g. salt concentration and acidity) and extrinsic factors in the case of storage or technological conditions (e.g. temperature, relative humidity, and gaseous atmosphere). The standard procedures used in food technology minimise the

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microbiological hazards during food production as shown in the review by AANTREKKER *et al.* (2003). However, a food product may be exposed to environmental variations during purchase, e.g. shopping, transport, food cooling, etc. In this way, it is important to be able to estimate the risk of undesirable microorganism growth, such as *Listeria* sp. at low temperatures (McKELLAR *et al.* 1997; PANISELLO & QUANTICK 1998; CHEROUTRE-VIALETTE & LEBERT 2000), halophilic pathogens such *Vibrio parahaemolyticus* (MILES *et al.* 1997), or thermophilic bacteria *Bacillus* sp. that survive the pasteurisation procedure.

As discussed in the paper of MURPHY *et al.* (1999) the dairy industry relies heavily on the pasteurisation process as a means of product safety assuring. However, thermophilic bacteria, particularly aerobic species capable of forming endospores with a high resistance to heat, are largely unaffected by this level of heat treatment. During the manufacture of milk powder, the surfaces of heat exchangers provide conditions favourable for the growth of thermophiles. Other potential sites for growth may occur due to the evaporator effects because the applied vacuum limits the temperatures to 40–70°C that are most favourable for thermophilic growth. Recently, JANSTOVA and LUKASOVA (2001) compared the heat resistance of the spores of 58 strains of *Bacillus* spp. that were isolated from raw milk (bulk tank milk and milk from individual cows) and farm environments (faeces, silage, litter, swabs from skin and udder). The authors compared the survival of spores in heat-treated dairy products. The isolates of *Bacillus* spp. were tested for thermoresistance within the temperature range of 95–135°C and various exposure times. The highest thermoresistance was found with *B. licheniformis* spores which survived the temperature of 135°C. The results indicate that some *Bacillus* spores may survive in milk even after the heat treatment.

This paper deals with a study of thermophilic bacterial colony growth within solid and wet matrices. The growth, structure, and physiological activities of thermophilic bacterial colonies were studied in the laboratory model system under defined conditions. The model selected in this paper is a medium solidified with agar. Cells grown as colonies within this agar matrix can be investigated using different techniques which include viable and total count determinations, light and electron microscopy, microelectrode studies or digital pho-

tography coupled to computer-aided analysis data. In order to generalise the results, the parameters of conventional mathematical model of predictive microbiology (McMEEKIN *et al.* 1993) were identified. For an extensive review of such models see e.g. VAN GERWEN and ZWIETERING (1998).

## MATERIAL AND METHODS

**Organisms.** Pure cultures of thermophilic bacterial strains – *Bacillus acidocaldarius* (CCM 3497), *Bacillus stearothermophilus* (CCMI 237), *Thermus aquaticus* (CCM 3488), *Thermus ruber* (CCM 4212), and *Thermus species* (CCM 4199) were used throughout our study. The original cultures were kept in Eppendorf probes (1 ml) with glycerol (2:1) at –58°C.

**Medium.** The following model solid matrix for thermophilic bacteria growth was used Luria-Bertani agar medium: casein hydrolysate 10 g, yeast extract 5 g, NaCl 5 g, agar 15 g, distilled water to 1 l, pH adjusted to 7.0 ± 0.2. The medium was sterilised at 121°C during 20 min.

**Growth conditions.** The hot and sterile agar medium was poured into plastic Petri dishes (diameter 8.5 cm) in a flowbox. After two days, the agar plates were inoculated using a sterile wooden stick. In order to prevent drying-out, the inoculated plates were covered with aluminium foil and were cultivated at 40, 50, 55, or 60°C.

**Analysis of colony growth.** The colony growth was recorded by means of digital camera Olympus model C-3000 ZOOM (Olympus, Japan), after: 12, 20, 28, 36, 44, 52, 60, 72, 84, 96, 108, 132 and 156 hours of cultivation. The interpretation of digital photographs was performed by means of the computer package LUCIA (LIM, CZ). It allowed to evaluate the surface and the apparent diameter of colonies and to estimate the segmentation of growing zones.

**Mathematical model description.** Although the cultivating of bacteria in colonies on the surface of a solid nutrient medium is a general experimental technique, the physiological laws of the colonial growth are not yet as commonly known as the laws and models that describe the growth in a submerged culture.

In predictive microbiology, we need to know which factors govern the rate of development of microbial contamination of food and what governs the ultimate size of the colony. Following the Pirt's idea (PIRT 1975), we assume that the basis on which the concepts of colony growth are built are the growth laws derived from the study of the

growth of populations of organisms in submerged homogeneous liquid cultures where all nutrients are present in excess and the growth inhibitors are not accumulated. In such cultures, the organisms grow exponentially

$$\frac{dN}{dt} = \mu N \quad (1)$$

where  $N$  is the number of cells,  $t$  is time and  $\mu$  is a parameter known as the “specific growth rate”, which is a direct measure of the growth rate of a population in a given environment. If the flows of nutrients and oxygen into a microbial colony are unrestricted and the growth inhibitors are not accumulated, the number of cells in the bacterial colony should conform to Eq. (1). Suppose the cells divide equimodally and the colony grows as a disc of constant height and radius. In such case, the radius of the colony should increase exponentially with time. But it is common experience that bacterial colonies of visible size growing on the surface of a solid nutrient do not spread outwards at an exponential rate and the observed increase of cell number or mass is not comparable with a submersed culture. PIRT (1975) developed a model that describes the growth of a bacterial colony on solid homogenous surface. After inoculation on agar plate, the bacterial cells receive nutrients at concentrations above the growth limiting values and, consequently, as shown in Figure 1(a), the colony will grow exponentially. In the exponential phase, it is concluded that the growth will occur equally in all directions on the surface and lead to a hemispherical shape of the colony. During this phase, a decrease of the nutrients content develops under the colony and finally stops the growth there. Then the growth of bacterial cells

will be restricted to the outer peripheral zone of the colony. This situation is represented in Figure 1(b). Due to the diffusion-limited flux of the substrate that covers the requirement for the maintenance energy, a dynamic equilibrium is established resulting in nearly a constant value of the height of the colony ( $h$ ) except the peripheral growing zone. Thus the colony growth is limited only to the growing zone on its rim of the width ( $w$ ). The growth dynamics according to this model can be derived in the following way: Let  $X$  be the total wet mass of the colony and  $X_G$  that one in the growing zone. We suppose that the specific growth rate  $\mu$  as defined by Eq. (1) remains constant, then we have to rewrite Eq. (1) as follows:

$$\frac{dX}{dt} = \mu X_G \quad (2)$$

If the width ( $w$ ) of the growing zone is small when compared to the colony diameter ( $D$ ), then we can approximate the value  $X_G$  by an expression  $\pi D h w \rho$ , where  $\rho$  is the density expressed as the mass of wet biomass per volume of the colony. In a similar way, the total mass of the colony  $X$  can be expressed as a function of the colony diameter by relation  $0.25 \pi D^2 h \rho$ . Thus, according to this simple model given by Eqs. (1) and (2), the colony diameter should increase exponentially at the beginning of cultivation:

$$\frac{dD}{dt} = \frac{\mu}{2} D; \quad D < 2w \quad (3)$$

and then linearly with time at constant radial growth rate equal to  $2\mu w$ .

$$\frac{dD}{dt} = 2\mu w; \quad D > 2w \quad (4)$$

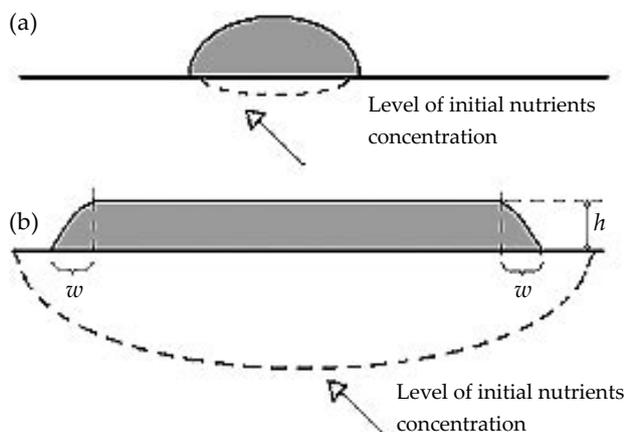


Figure 1. Vertical cross-sections through model of a colony: (a) during initial exponential of substrate unlimited growth; (b) during phase of constant radial growth rate. The area of width  $w$  represents the postulated zone of growth and  $h$  is the height of the colony

## RESULTS AND DISCUSSION

Figure 2(a) shows an example of the digital record of a bacterial colony growing at suboptimal temperature 60°C after 156 h. Two basic parameters that characterise the geometry of the colony (area and length of periphery) were evaluated by means of a computer. The blue region in Figure 2(b) shows the typical output of the computer program LUCIA. As the shape of the colony was not an ideal circle, the equivalent (apparent) diameter of the colony was calculated using the formula:

$$D_{\text{Apparent}} = 2 \sqrt{A/\pi} \quad (5)$$

As shown in Figure 2, the real length of the periphery estimated by means of box-counting algorithm has to be greater than the ideal value calculated as  $\pi D_{\text{Apparent}}$  due to the dendritic character of the colony growth (MATSUURA & MIYAZIMA 1993). In order to express this relation quantitatively, we introduced a measure of ruggedness ( $\Phi$ ) as the

ratio between the measured length of the periphery and its ideal value  $\pi D_{\text{Apparent}}$

$$\Phi = \text{length of periphery} / \pi D_{\text{Apparent}} \quad (6)$$

For a colony of ideal circular shape, the value  $\Phi$  equals 1. However, when colony grows irregularly and its rim is rugged, then Eq. (4) should be modified by multiplying its right side by  $\Phi$ .

MCMEEKIN *et al.* (1993) summarised the data on the temperature dependence of the growth of thermophilic bacteria *Bacillus* sp. and found that minimum temperature for the growth in a submerged culture is close 20°C, optimum at 51°C, and the upper threshold limit for surviving is 62°C. For the growth of *Thermus aquaticus*, it was reported that the lower threshold limit of temperature is 20°C, optimum can be found between 70–72°C, and the upper limit of surviving is near 79°C (FUJIO & KUME 1991). However, during our experiments the thermophilic bacteria

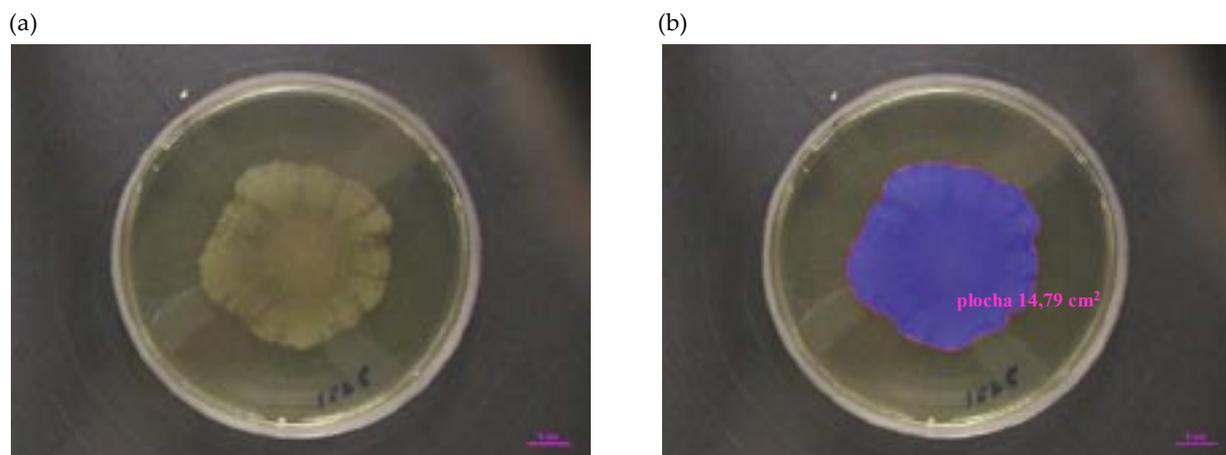


Figure 2. An example of a colony formed by thermophilic bacteria after 156 h of cultivation at 60°C (a) recorded by the digital camera. Evaluation of the digital photography by a computer program LUCIA (b)

Table 1. Kinetic parameters characterising the growth of bacterial colony at 55°C described by Eqs. (4) and (6)

Strain	$2\omega\mu\Phi$ (mm/h)	$\Phi$ (-)	$r^2$ (-)
<i>Bacillus stearothermophilus</i> (CCMI 237)	0.025	1.31	0.99
<i>Bacillus acidocaldarius</i> (CCM 3497)	0.017	1.58	0.87
<i>Thermus aquaticus</i> (CCM 3488)	0.010	1.19	0.92
<i>Thermus ruber</i> (CCM 4212)	0.074	1.30	0.96
<i>Thermus species</i> (CCM 4199)	0.028	1.32	0.96

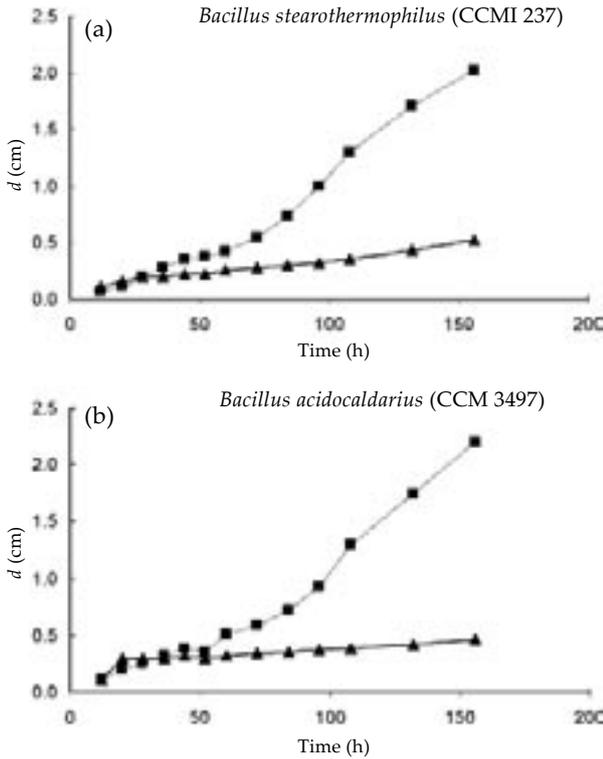


Figure 3. Time dependence of apparent diameter of the colony of (a) *Bacillus acidocaldarius* (CCM 3497) and (b) *Bacillus stearothermophilus* (CCMI 237) cultivated at 55°C (▲) and 60°C (■)

revealed no ability to grow in colonies at 40 and 50°C. The exception to this rule was the strain *Thermus species* (CCM 4199) which formed small colonies of the area of  $0.16 \pm 0.06 \text{ cm}^2$  after 156 h of cultivation. In the temperature range close to the usually cited optimum, an important growth of thermophilic bacteria in colonies was detected. Figures 3 and 4 summarise the time dependence of the apparent colony diameter calculated by means of Eq. (5).

It is evident that for the temperature of 55°C the course of the experimental data follows the linear dependence (Eq. 4). Using linear regression, we estimated the value of parameter  $2\mu w$  as a slope of the line that fitted the data. The value of the measure of ruggedness ( $\Phi$ ) was symmetrically distributed in the neighbourhood of the mean during the whole experiment that was performed at 55°C. The results are summarised in Table 1.

However, as shown in Figures 3 and 4, the law of linear growth is not strictly valid for the cultivation temperature of 60°C as in the previous case. With the exception of *Thermus ruber* (CCM 4212), we could observe an acceleration of the growth after two days of cultivation. This phenomenon is not typical for the growth of bacterial colonies on a solid surface (PIRT 1975) because the later phase of cul-

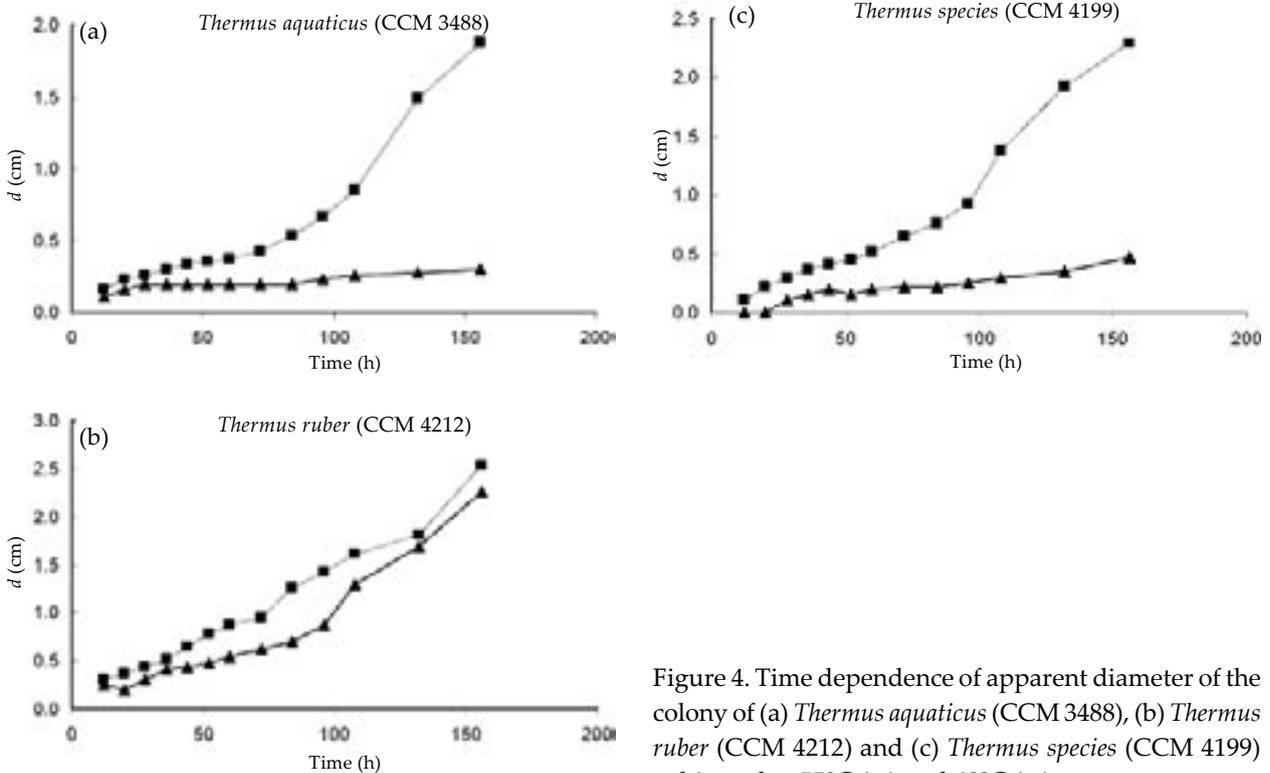


Figure 4. Time dependence of apparent diameter of the colony of (a) *Thermus aquaticus* (CCM 3488), (b) *Thermus ruber* (CCM 4212) and (c) *Thermus species* (CCM 4199) cultivated at 55°C (▲) and 60°C (■)

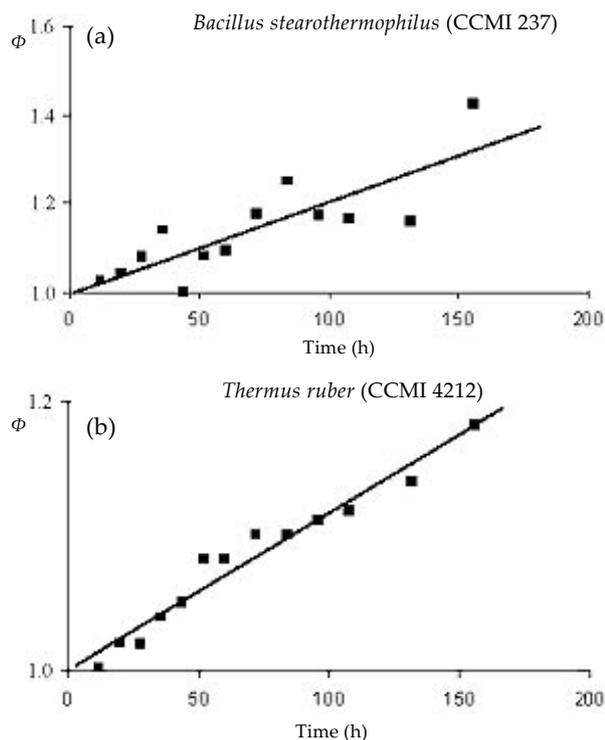


Figure 5. Time dependence of a measure of ruggedness ( $\Phi$ ) defined by Eq. (6) and cultivation time (h) for (a) *Bacillus stearothermophilus* (CCMI 237) and (b) *Thermus ruber* (CCM 4212) cultivated at 60°C

tivation is usually linked to the growth rate decay. According to our model (Eq. 4), we can attribute this unexpected increase in the colony diameter either to an increase of the specific growth rate  $\mu$  or to an increase of the length of the growing zone  $w$ . Knowing that there is no apparent physiological reason for an increase of the value  $\mu$ , we pay our attention to the parameter  $w$ . The value of the length of the growing zone can increase when the colony cease to form an ideal circle and the microbes on its rim begin to proliferate in branches to produce a dendrite shape. This morphological differentiation can be quantitatively described by the measure of ruggedness ( $\Phi$ ) that was introduced above [see Eq. (6)]. Figure 5 show the typical course of  $\Phi$  during the cultivation of *Bacillus* sp. and *Thermus* sp. When compared with the experiment at 55°C, a

linear relationship exists here between  $\Phi$  and the cultivation time. Substituting this linear relationship into Eq. (4), we receive, after integration, the following quadratic equation describing the effect of irregular proliferation of the colony:

$$D_{\text{Apparent}}(t) = 2\mu wt(1 + ct) \quad (7)$$

where  $c$  describes the linear increase of the colony rim ruggedness as a function of the cultivation time ( $t$ ). Table 2 summarises the fit of Eq. (7) with the experimental data. The correlation between the model and the data is very high.

In conclusion, we would like to compare our results with those concerning other representative bacteria causing food spoilage or poisoning. PIRT (1975) summarised the data on the colony growth

Table 2. Kinetic parameters characterising the growth of bacterial colony at 60°C described by Eq. (7)

Strain	$2w\mu$ (mm/h)	$c \cdot 10^3$ (1/h)	$r^2$ (-)
<i>Bacillus stearothermophilus</i> (CCMI 237)	0.024	11.9	0.99
<i>Bacillus acidocaldarius</i> (CCM 3497)	0.023	13.1	0.99
<i>Thermus aquaticus</i> (CCM 3488)	0.015	18.3	0.98
<i>Thermus ruber</i> (CCM 4212)	0.068	0.8	0.99
<i>Thermus species</i> (CCM 4199)	0.028	10.9	0.99

of *Escherichia coli* and found that the diameter of colonies increased linearly with time. The values of parameter  $2w\mu$  for this bacteria growing at optimal temperature 37°C range between 0.02–0.08 mm/h. *Listeria monocytogenes* with its optimal growth temperature is a typical representant of cryophilic food contaminants. Based on recent data published by BARAKAT and HARRIS (1999) and AUGUSTIN *et al.* (2000), we estimated the value of parameter  $2w\mu$  to be between 0.04–0.08 mm/h. Comparing Tables 1 and 2, we were surprised that the rate of the colony growth of thermophilic bacteria near the optimal temperature was practically the same. This finding may be very important for predictive microbiology in food processing. Knowing the temperature and duration of certain unit operations, we can estimate qualitatively and quantitatively what kind of bacteria can, near its optimal growth temperature, colonise the surface of food.

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### Souhrn

MELZICH K., VOTRUBA J., SEKAVOVÁ B., PITERKOVÁ L., RYCHTERA M. (2004): **Růst kolonií termofilních bakterií a jeho důsledky v potravinářském průmyslu**. *Czech J. Food Sci.*, **22**: 1–8.

Byla studována kinetika růstu termofilních bakterií *Bacillus acidocaldarius* (CCM 3497), *Bacillus stearothermophilus* (CCMI 237), *Thermus aquaticus* (CCM 3488), *Thermus ruber* (CCM 4212) a *Thermus species* (CCM 4199) na pevných substrátech při teplotách od 40 do 60 °C. Nárůst bakteriálních kolonií na agarových plotnách byl dokumentován pomocí digitálního fotoaparátu po dobu 160 hodin; tato data byla zpracována metodami analýzy obrazu. Bylo

zjištěno, že při nižších teplotách průměr kolonie roste přímo úměrně s časem a k jeho výraznému zrychlení dochází při suboptimální teplotě 60 °C. Rovněž byl navržen jednoduchý matematický model popisující neobvyklý fenomén, a to nepravidelný růst okraje kolonie.

**Klíčová slova:** termofilní bakterie; růst kolonií; prediktivní modelování; kažení potravin; činnost mikroorganismů; analýza obrazu

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